APPENDIX G

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Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development

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ABSTRACT **We have recently cloned the human fms-like tyrosine kinase 4 gene *FLT4*, whose protein product is related to two vascular endothelial growth factor receptors FLT1 and KDR/FLK1. Here the expression of *FLT4* has been analyzed by *in situ* hybridization during mouse embryogenesis and in adult human tissues. The FLT4 mRNA signals first became detectable in the angioblasts of head mesenchyme, the cardinal vein, and extraembryonally in the allantois of 8.5-day postcoitus (p.c.) embryos. In 12.5-day p.c. embryos, the FLT4 signal decorated developing venous and presumptive lymphatic endothelia, but arterial endothelia were negative. During later stages of development, FLT4 mRNA became restricted to vascular plexuses devoid of red cells, representing developing lymphatic vessels. Only the lymphatic endothelia and some high endothelial venules expressed FLT4 mRNA in adult human tissues. Increased expression occurred in lymphatic sinuses in metastatic lymph nodes and in lymphangioma. Our results suggest that FLT4 is a marker for lymphatic vessels and some high endothelial venules in human adult tissues. They also support the theory on the venous origin of lymphatic vessels.**

The physiology of the vascular system, embryonic vasculogenesis and angiogenesis, blood clotting, wound healing, and reproduction as well as several diseases involve the vascular endothelium lining the blood vessels (1, 2). In the mouse embryo, certain mesenchymal cells differentiate into endothelial cell precursors *in situ*—e.g., in the head mesenchyme, in the dorsal aorta, and in the cardinal veins (3–5). Blood islands of the yolk sac are sites of extraembryonic vasculogenesis (6, 7). Further development of the vascular tree occurs through angiogenesis (8, 9). According to some theories, the formation of the lymphatic system starts shortly after arterial and venous development by venous sprouting (10, 11).

After the fetal period endothelial cells proliferate very slowly, except during angiogenesis associated with neovascularization (12, 13). Among the factors stimulating angiogenesis, the acidic and basic fibroblast growth factors (FGF-1 and FGF-2) and the vascular endothelial growth factor (VEGF) exert their effects via specific cell surface receptor tyrosine kinases: FGF receptor 1 and the endothelial-specific fms-like tyrosine kinase 1 (FLT1) and KDR/FLK1 receptors, respectively (refs. 14–16; for review, see ref. 17). The protein product of the FLT4 receptor tyrosine kinase cDNA is structurally similar to the FLT1 and KDR/FLK1 receptors (18), but FLT4 does not bind VEGF (19). Here we have analyzed FLT4 mRNA expression during vasculogenesis and angiogenesis in mouse embryos and in endothelia of human adult tissues in normal and pathological conditions.

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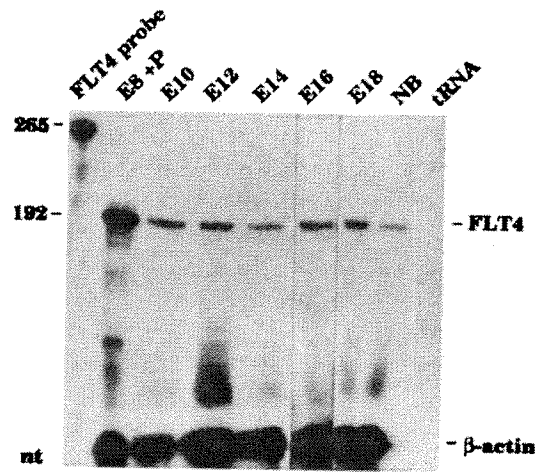

FIG. 1. RNase protection analysis of RNA isolated from mouse embryos of various gestational ages (E8.5–E18.5) and from a newborn mouse. Sample E8+P also contains the placenta. The size of the probe and the protected FLT4 fragment are given in nt; β-actin was used as a control. NB, newborn; E, embryonic day.

MATERIALS AND METHODS

Analysis of RNA in Mouse Tissues. From a subcloned FLT4 DNA fragment of a λ FIX II genomic library from 129SV mice (Stratagene) PCR was used to amplify and clone exon fragments covering nt 1–192 and 1745–2049 of mouse FLT4 cDNA (20). Total RNA was isolated and analyzed by RNase protection (21) with probes corresponding to nt 1–192 of FLT4 cDNA and 1188–1279 of β-actin cDNA (22). *In situ* hybridization of sections was performed as described (23) and the mouse Tie 1C1D plasmid was used as a control (24).

Analysis of RNA in Human Cells and Tissues. Endothelial cells from humans were isolated, cultured for five to eight passages, and used for isolation of polyadenylylated RNA as described (25, 26). Human tissues sent for routine histopathological diagnosis were used for RNA *in situ* hybridization. Probes covered bp 1–595 of the human *FLT4* cDNA (18), 1–2334 of the von Willebrand factor cDNA (27), and 1–2190 of the *Tie* receptor cDNA (28). The normal *in situ* protocol did not work for human tissue samples routinely fixed in 10% formalin. However, when the proteinase K digestion step was replaced with microwave treatment, specific hybridization signals were obtained (29, 30).

RESULTS

Analysis of FLT4 mRNA in Early Mouse Embryos. Analysis of RNAs collected during different phases of mouse develop-

§Deceased February 13, 1994.
Abbreviations: p.c., postcoitus; HEV, high endothelial venule.

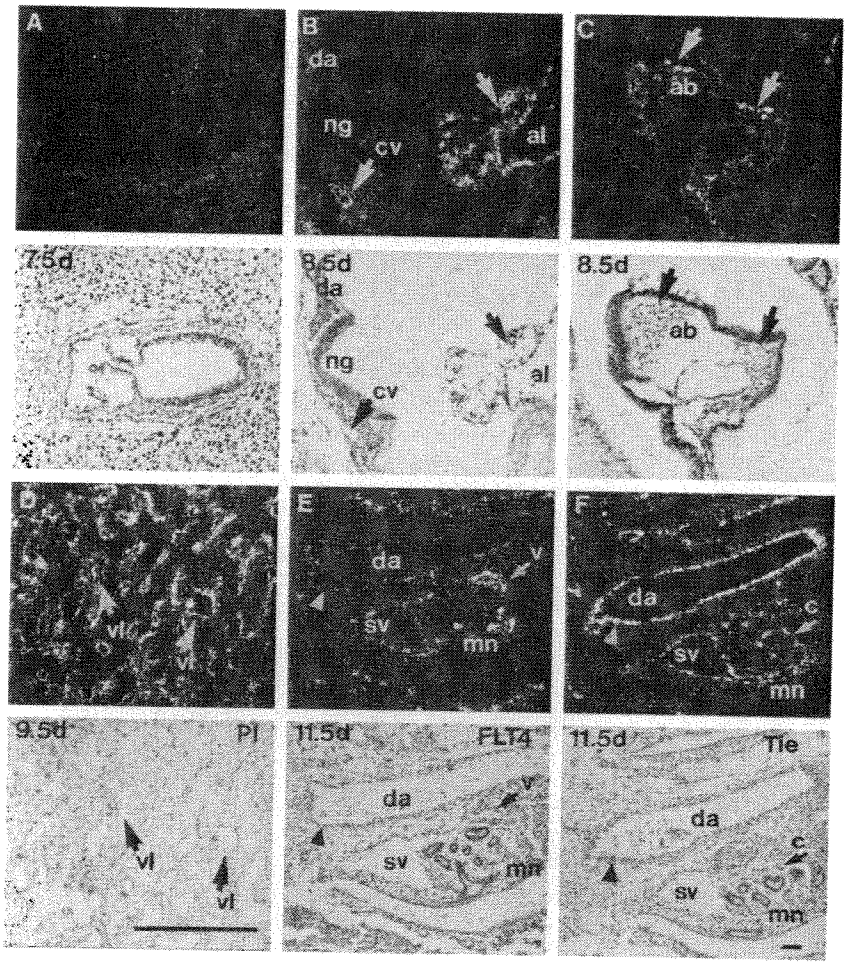

FIG. 2. Expression of FLT4 mRNA in 7.5-, 8.5-, and 11.5-day p.c. embryos. Dark-field and bright-field photomicrographs of *in situ* autoradiograms are shown. No expression of FLT4 mRNA is detected in a 7.5-day p.c. embryo (*A*). FLT4 expression in an 8.5-day p.c. mouse embryo is shown in *B* and *C*. Arrows indicate FLT4-positive cells in the endothelium of posterior cardinal vein (cv), in the allantois (al) in *B* and in angioblasts (ab) of the head mesenchyme in *C*. In a 9.5-day p.c. placenta, FLT4 transcripts can be seen in endothelial cells of venous lacunae (vl) (*D*). (*E* and *F*) Comparison of FLT4 and Tie hybridization signals in 11.5-day p.c. embryos. The region of the developing dorsal aorta (da) (arrowheads) and metanephros (mn) is shown. (×12.) Note that the da is negative for FLT4 but positive for Tie mRNA, whereas both probes hybridize with the endothelium of the subcardinal vein (sv). Also, the FLT4 probe gives a signal from the metanephric vein (v), whereas the Tie probe mostly hybridizes with the developing metanephric capillaries (c, arrows). ng, Neural groove. (Bar = 30 μm.)

ment by RNase protection assay showed that FLT4 mRNA was expressed throughout embryogenesis from day 8.5 postcoitus (p.c.) to newborn mice without variations in signal intensity (Fig. 1). Placenta contained enhanced amounts of FLT4 mRNA (lane E8+P; data not shown).

To localize FLT4 transcripts to specific cells and tissues, sections of 7.5- and 8.5-day p.c. mouse embryos were hybridized with labeled FLT4 probes. As shown in Fig. 2, FLT4 mRNA was not expressed in 7.5-day p.c. mouse embryos (Fig. 2*A*), but abundant signals were detected in the posterior cardinal vein and in the angioblasts of the head mesenchyme on day 8.5 of development (Fig. 2 *B* and *C*). In contrast, the dorsal aorta and developing heart (data not shown) did not express FLT4 mRNA. In the extraembryonic tissues, FLT4 was prominent in the allantois (Fig. 2*B*), whereas developing blood islands of the yolk sac were negative (data not shown). In the developing 8.5-day p.c. placenta, FLT4 signal was seen in peripheral sinusoidal veins and in the endothelium of venous lacunae (Fig. 2*D*). Also, giant cells partially fused to the Reichert's membrane (data not shown) expressed FLT4 mRNA.

Thus, although FLT4 expression was very prominent in the earliest endothelial cell precursors, the angioblasts, it appeared to be restricted only to certain vessels of 8.5-day p.c. embryos. This is clear from the comparison of FLT4 with the Tie receptor,

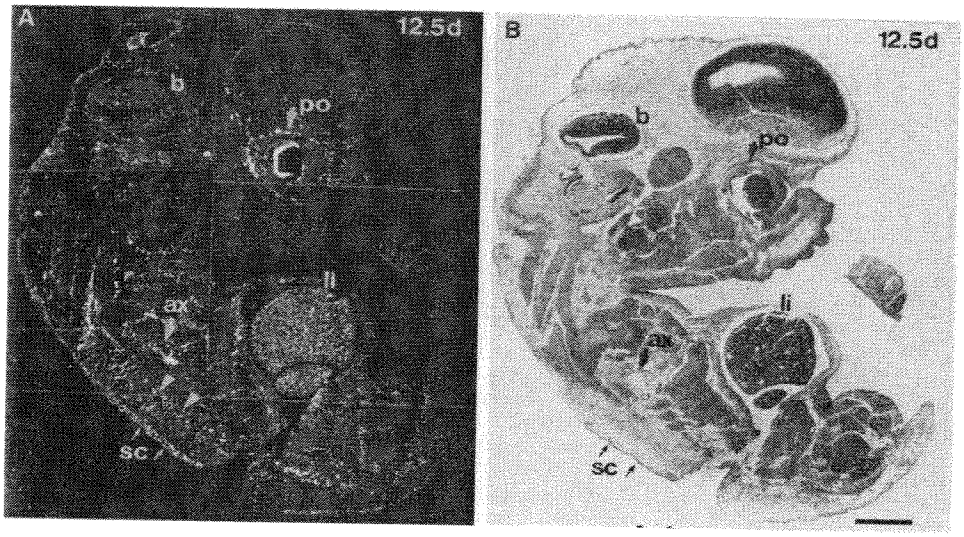

FIG. 3. FLT4 mRNA expression in a 12.5-day p.c. embryo. A parasagittal section through the axillar plane is shown. Note that FLT4 mRNA is prominent in dilated vessels of the axilla (ax), in a plexus-like pattern in the periorbital (po) region, in the paravertebral tissue (arrowheads), and in the subcutaneous (sc) tissue. b, Brain; li, liver. (Bar = 5 μm.)

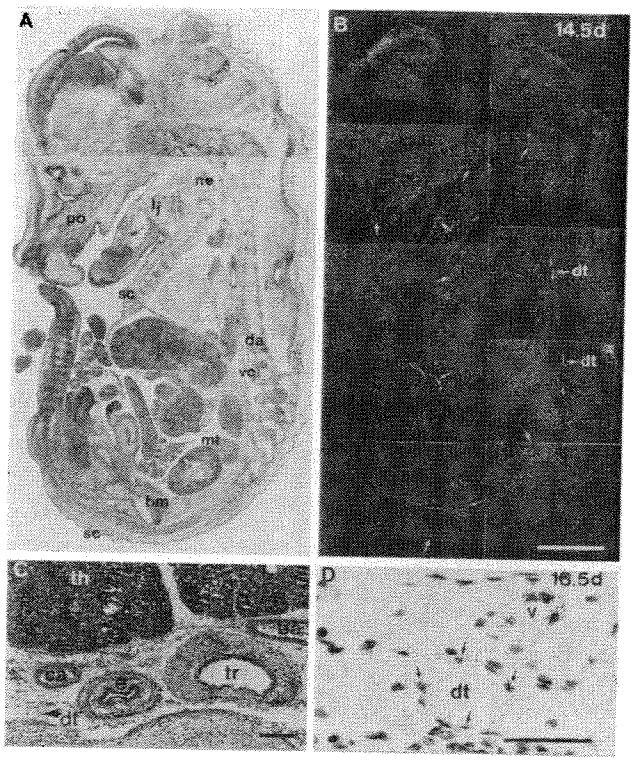

FIG. 4. FLT4 in 14.5- and 16.5-day p.c. embryos. (*A* and *B*) Bright-field and dark-field images of a midsagittal section. (*C*) Transverse section of a 16.5-day p.c. embryo with hematoxylin and eosin staining. (*D*) Higher magnification of the region of ductus thoracicus; autoradiographic grains can be seen over the endothelial cells. (×20.) Also, the small vessel (v) in the upper part of the photograph is positive. da, Dorsal aorta; vc, inferior vena cava; bm, bone marrow; po, periorbital region; lj, lower jaw; ne, neck region; sc, subcutis; mt, mesenterium; dt, thoracic duct; th, thymus; tr, trachea; e, esophagus; ca, carotid artery; ba, brachiocephalic artery. (*A*–*C*, bar = 10 μm; *D*, bar = 1 mm.)

which is known to be expressed in all endothelial cells of developing mouse embryos and thus provides a marker for these cells (31, 32). Notably, in contrast to the Tie probe, the FLT4 probe hybridized very weakly if at all with arterial endothelia of 11.5-day p.c. embryos—e.g., with the endothelium of the developing dorsal aorta (Fig. 2 *E* and *F*) or the carotid arteries (data not shown). Instead, the FLT4 signal was much more prominent in the developing veins. For example, FLT4 signal was detected in anterior cardinal veins in the neck area (data not shown) and in veins surrounding the developing metanephros (Fig. 2*E*),

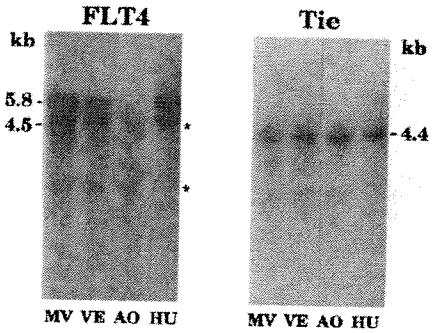

FIG. 5. Comparison of FLT4 and Tie mRNA expression in cultured endothelial cells. Northern blot analysis of polyadenylylated RNA from human foreskin microvascular (MV), femoral vein (VE), aortic (AO), and umbilical vein (HU) endothelial cells. For comparison, the hybridization signal of the Tie receptor tyrosine kinase mRNA is shown. Bands resulting from nonspecific binding of the probe to the rRNA are marked with asterisks.

whereas the Tie probe predominantly recognized developing capillaries within the metanephros (Fig. 2*F*).

FLT4 mRNA in 12.5-Day p.c. Embryos. Fig. 3 illustrates FLT4 signals in a parasagittal section of a 12.5-day p.c. mouse embryo. FLT4 mRNA is distributed in several regions of the embryo, being particularly prominent in a dilated vessel of the axillar region (Fig. 3*A*). Similar FLT4-positive vessel networks were seen in the midsagittal section in the jugular area (data not shown). A plexus-like pattern of FLT4-expressing vessels appeared in the periorbital region and surrounding the developing vertebrae. Also, just beneath the developing skin, an FLT4-positive vascular network was evident. Weaker capillary signals were obtained from several regions, including the developing brain. FLT4 mRNA could also be detected in small vessels of the neck region, of the developing snout, and at the base of the developing tongue as well as in the tail region. In addition, the liver was strongly positive for FLT4 mRNA, which occurred in a spot-like pattern.

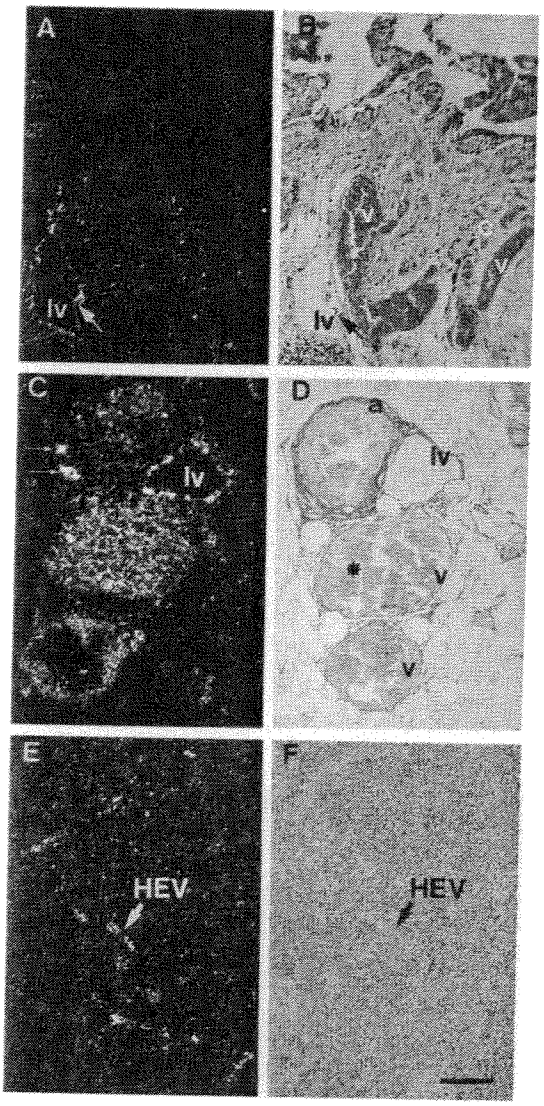

FIG. 6. FLT4 in adult human lymphatic vessels of the lung (*A* and *B*), mesenterium (*C* and *D*), and tonsil (*E* and *F*). Note that only the lymphatic vessels (lv) in *A* and *C* give an FLT4 signal, whereas the veins (v), capillaries (c), and arteries (a) are negative for FLT4 mRNA. Signal within these vessels results from reflection of light from the red cells, which are seen in an adjacent stained section (asterisk in *D*). Arrows in *C* indicate small lymphatic vessels. In the tonsil, the signal is observed in the endothelia of some HEVs. (Bar = 200 μm.)

FLT4 mRNA in 14.5- and 16.5-Day p.c. Embryos. During further development, FLT4 mRNA appeared to become more restricted to certain vessels of the embryo. A 14.5-day p.c. embryo clearly shows this restricted pattern of expression (Fig. 4 *A* and *B*). In the midsagittal section of Fig. 4, the most prominent FLT4 signal is seen along the developing vertebral column on its anterior side. This signal seems to originate from endothelial cells of the thoracic duct. In contrast, the dorsal aorta and inferior vena cava were negative. Dilated vessels in the mesenteric region were also positive for FLT4. Furthermore, as in the 12.5-day p.c. embryos, vessel networks along anatomical boundaries in the periorbital, lower jaw, as well as in the neck region contained FLT4-expressing endothelia. Similar structures were present in the pericardial space and throughout the subcutaneous tissue. Notably, in contrast to FLT4-negative vessels, all FLT4-positive vessels were devoid of red cells in their lumen. This expression pattern suggested that FLT4 becomes confined to the endothelium of lymphatic vessels at this time of development. An additional site where we observed FLT4 expression was the sinusoidal endothelium of the developing bone marrow.

Photographs of a transverse section of the upper thorax of a 16.5-day p.c. embryo hybridized with the FLT4 probe are shown in Fig. 4 *C* and *D*. Higher magnification of the region of the thoracic duct is shown in Fig. 4*D*, where the FLT4 autoradiographic grains can be seen. Endothelial cells of the thoracic duct as well as a small vessel in the vicinity hybridize with the FLT4 probe.

Analysis of FLT4 mRNA in Human Endothelial Cells *in Vitro* and *in Vivo*. Reflecting the selective FLT4 expression in early embryos, cultured human microvascular, venous, and umbilical vein endothelial cells were positive for the FLT4-specific 5.8- and 4.5-kb mRNAs (33), whereas the aortic endothelial cells were negative (Fig. 5). In contrast, *Tie*, another endothelial receptor tyrosine kinase gene was expressed as a 4.4-kb mRNA in all endothelial cell types studied.

We also studied FLT4 in adult human tissues by using the human FLT4 probe. In the lung, mesenterium, and appendix, lymphatic endothelia gave FLT4 signals, while veins, arteries, and capillaries were negative (Fig. 6 *A–D*; data not shown). To determine whether FLT4 is expressed in the high endothelial venules (HEVs), the tonsils were studied. FLT4-specific autoradiographic grains were detected in some, but not all, HEVs in these tissues (Fig. 6 *E* and *F*).

Analysis of FLT4 mRNA in Normal and Metastatic Lymph Nodes and in Lymphangioma. In Fig. 7 a portion of a human mesenteric lymph node is analyzed for FLT4 expression. FLT4 mRNA was present in the lymphatic sinuses and afferent and efferent lymphatic vessels (data not shown). The same pattern is seen in lymph nodes containing adenocarcinoma metastases (Fig. 7 *C* and *D*). Some HEVs in both normal and metastatic lymph nodes were also positive. However, the lymphatic endothelium of metastatic lymph nodes gave an enhanced signal. In Fig. 7*E*, a strong FLT4 mRNA expression is shown in a cystic lymphangioma (compare with the hematoxylin-stained section in Fig. 7*G*). Notably, the specific expression of FLT4 in lymphatic endothelium is evident from comparison with the *in situ* signals for von Willebrand factor mRNA in all blood vessels (Fig. 7*F*).

DISCUSSION

We have shown earlier that the expression pattern of FLT4 in comparison to FLT1 and KDR differs greatly in tissues of 18-week-old human fetuses (23). To understand the role of FLT4 during development, we cloned mouse cDNA probes for FLT4. Using these probes in *in situ* hybridization, we analyzed FLT4 expression during mouse development and confirmed the relevance of our findings in normal and pathological adult human tissues.

Like the *FLK1*, *FLT1*, *TIE*, and *TEK* endothelial receptor tyrosine kinase genes, the *FLT4* gene was not expressed in 7.5-day p.c. embryos. The results of RNase protection suggested that the relative expression level is quite stable after 8.5 days of mouse development. The earliest signals appeared in the angioblasts of the head mesenchyme and veins of the embryo. In contrast, the dorsal aorta, endocardium of the heart, and blood islands of the yolk sac were negative, unlike for FLK1, FLT1, Tie, and Tek mRNAs (refs. 31 and 34; unpublished data). The restriction of FLT4 expression to the venous system was even more clear in samples from 11.5-day p.c. mouse embryos, where Tie mRNA was expressed also in arteries.

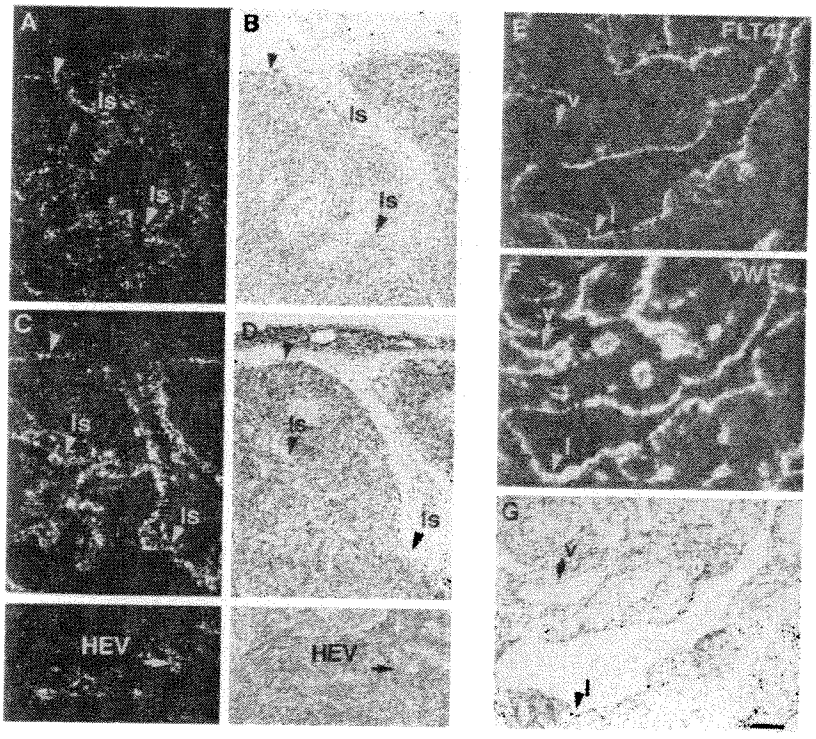

FIG. 7. FLT4 mRNA in normal (*A* and *B*) and metastatic (*C* and *D*) lymph node and in lymphangioma (*E–G*). Arrowheads mark lymphatic sinuses (ls) and HEVs, which are FLT4 positive. A comparison of FLT4 and von Willebrand factor (vWF) signals shows both in the lymphatic endothelium (l) but only von Willebrand factor signal in the capillary and venous (v) endothelia. (*A–D*, bar = 10 μm; *E–G*, bar = 100 μm.)

The idea of lymphatic expression arose during the study of 14.5- and 16.5-day p.c. embryos, because at that stage large arteries and veins as well as small vessels containing red blood cells had no FLT4 signal in their endothelium. However, lymphatic vessels were difficult to identify, because no specific markers are available for them. Since the beginning of the 20th century, three different theories concerning the origin of the lymphatic system have been presented (11, 35, 36). The most widely accepted theory was proposed by Florence Sabin (10), who suggested that during the fetal period lymphatic structures sprout from large central veins in certain locations to form the primordial lymph sacs. Subsequently, the sacs enlarge, coalesce, and form new sprouts, which grow into the periphery of the embryo (11, 12, 35, 36).

The expression of FLT4 in developing mouse embryos coincides with Sabin's model of lymphatic development. According to her theory, the jugular sacs develop from anterior cardinal veins in the neck, and all other lymphatic sacs develop by sprouting from the mesonephric vein and veins in the dorsomedial edge of the Wolffian bodies (36). We found very prominent FLT4 signals in the venous compartment, in the anterior cardinal veins, and mesonephric vein in 11.5-day p.c. embryos, and a day later we could detect the first FLT4-positive sac-like structures in the jugular and axillary areas. The main lymphatic duct, the thoracic duct, is formed by the union of duct primordia (36). Fig. 4 shows such primordia in a 14-day p.c. embryo and a transverse section of the 16.5-day p.c. embryo shows FLT4 signal in the thoracic duct.

At least some of the specificity of FLT4 expression was retained in cultures of human endothelial cells and *in situ* hybridization analysis of adult human tissues confirmed the restriction of FLT4 to the lymphatic system. FLT4 expression was seen in the lymphatic endothelia and in sinuses of human lymph nodes. Interestingly, some of the HEVs, which have a cuboidal endothelium, shown to function in the trafficking of leukocytes to the lymph nodes, were FLT4 positive. FLT4 was also very prominent in lymphangiomas, which are benign tumors composed of connective tissue stroma, and growing endothelium-lined lymphatic channels (37). FLT4 mRNA was restricted to the lymphatic endothelium of these tumors and absent from their arteries, veins, and capillaries. In human lung, we were able to identify lymphatic vessels, which were the only FLT4-positive vessels in this tissue.

In conclusion, our present results show that the uniform venous and capillary expression of FLT4 becomes restricted to lymphatic vessels during mouse development, and in human adult tissues FLT4 is specifically expressed by lymphatic vessels and some HEVs. These results support the theories of the venous origin of lymphatic vessels. The major function of the lymphatic system is to provide fluid return from tissues and to transport many extravascular substances back to the blood (38). In addition, during the process of maturation, lymphocytes leave the blood, migrate through lymphoid organs and other tissues, and enter the lymphatic vessels, from which they return to the blood through the thoracic duct. Specialized venules, HEVs, bind lymphocytes again and cause their extravasation into tissues. Besides providing a marker for lymphatic vessels and some HEVs in human adult tissues, FLT4 may be actively involved in the genesis and maintenance of the lymphatic vessels.

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Expression of the Vascular Endothelial Growth Factor C Receptor VEGFR-3 in Lymphatic Endothelium of the Skin and in Vascular Tumors

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It is difficult to identify lymph vessels in tissue sections by histochemical staining, and thus a specific marker for lymphatic endothelial cells would be more practical in histopathological diagnostics. Here we have applied a specific antigenic marker for lymphatic endothelial cells in the human skin, the vascular endothelial growth factor receptor-3 (VEGFR-3), and show that it identifies a distinct vessel population both in fetal and adult skin, which has properties of lymphatic vessels. The expression of VEGFR-3 was studied in normal human skin by *in situ* hybridization, iodinated ligand binding, and immunohistochemistry. A subset of developing vessels expressed the VEGFR-3 mRNA in fetal skin as shown by *in situ* hybridization and radioiodinated vascular endothelial growth factor (VEGF)-C bound selectively to a subset of vessels in adult skin that had morphological characteristics of lymphatic vessels. Monoclonal antibodies against the extracellular domain of VEGFR-3 stained specifically endothelial cells of dermal lymph vessels, in contrast to PAL-E antibodies, which stained only blood vessel endothelia. In addition, staining for VEGFR-3 was strongly positive in the endothelium of cutaneous lymphangiomatosis, but staining of endothelial cells in cutaneous hemangiomas was weaker. These results establish the utility of anti-VEGFR-3 antibodies in the identification of lymphovascular channels in the skin and in the differential diagnosis of skin lesions involving lymphatic or blood vascular endothelium. *(Am J Pathol 1998, 153:395–403)*

Angiogenesis, the formation of new blood vessels from vascular endothelium, is a key event in several biological processes, including wound healing and tumor development.[1] The regulation of angiogenesis depends on a balance between stimulatory and inhibitory factors affecting the proliferation and differentiation of endothelial cells.[2] Vascular endothelial growth factor (VEGF), which belongs to the platelet-derived growth factor family, is currently known as the major inducer of angiogenesis and vessel permeability.[3] Other members of the family, closely related to VEGF, include PlGF, VEGF-B, VEGF-C, and VEGF-D.[4–9]

The biological activities of VEGF and VEGF-C are exerted via binding to tyrosine kinase receptors. Selective binding of these factors occurs to VEGF receptor (VEGFR)-1 (Flt-1) and VEGFR-3 (Flt4), respectively, and both of the factors also bind to VEGFR-2 (Flk-1/KDR).[5,6,10–15] The recently identified specific receptor for VEGF-B is VEGFR-1 (B. Olofsson et al, manuscript in preparation), whereas VEGF-D binds both VEGFR-2 and VEGFR-3.[7]

In the skin of transgenic mice, overexpression of the VEGF-C cDNA has been shown to selectively induce lymphatic endothelial cell proliferation and hyperplasia of the lymphatic vasculature.[16] Furthermore, in differentiated chick chorioallantoic membrane, purified mature VEGF-C also induced growth of lymphatic vessels, having very little effect on blood capillaries.[17] In the present work, we have analyzed the binding of VEGF-C and expression of VEGFR-3 in adult human skin, in cutaneous lymphangiomatosis and hemangioma samples using iodinated ligand binding, *in situ* hybridization, and immunohistochemistry for the identification of the specific receptors.

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Materials and Methods

In Situ *Hybridization*

Skin from 17- and 18-week-old human fetuses was obtained from legal abortions induced with prostaglandins. The gestational age was confirmed from the foot length.[18] The study was approved by the Ethical Committee of the Helsinki University Hospital. The skin samples were fixed in 4% paraformaldehyde for about 20 h before dehydration and paraffin embedding. The human antisense and sense VEGFR-3 RNA probes were generated from linearized pBluescriptIISK$^+$ plasmid (Stratagene, La Jolla, CA), containing an *Eco*RI-*Sph*I fragment corresponding to nucleotides 1 through 595 of the human VEGFR-3 (Flt4) cDNA.[12] Radiolabeled RNA was synthesized using T3 and T7 polymerases and ^{35}S-labeled UTP (Amersham Corp., Arlington Heights, IL). VEGFR-2 RNA probe was generated from linearized pBluescriptIISK$^+$ plasmid containing an *Eco*RI-*Hin*dIII fragment covering bp 6 through 715.[15] *In situ* hybridization of the paraffin sections was performed as described previously.[19] Alkaline hydrolysis was omitted for the VEGFR-3 probe. The high-stringency wash was for 90 minutes at 65°C in 1× standard saline citrate containing 30 mmol/L dithiothreitol. The slides were exposed for 4 weeks, developed, and stained with hematoxylin. Control hybridizations with sense strand did not give a specific signal above background.

Iodinated Growth Factor Binding

Recombinant human (rh) VEGF165 or the 21-kd mature form of VEGF-C was labeled with ^{125}I using the Iodo-Gen reagent (Pierce, Rockford, IL) and purified by gel filtration on PD-10 columns (Pharmacia, Uppsala, Sweden). The specific activities were 2.2×10^5 cpm/ng and 1.0×10^5 cpm/ng for rh-VEGF and rh-VEGF-C, respectively. The iodinated growth factors were tested for specific binding using PAE-VEGFR-1 and PAE-VEGFR-3 cells[20] and soluble receptor-immunoglobulin proteins.[7]

The skin samples obtained were frozen immediately and kept at −70°C. Frozen sections were cut at 7 μm and then mounted onto silane-coated slides and stored in airtight boxes at −70°C. After thawing, the sections were incubated for 30 minutes at room temperature in the blocking solution, (minimum essential medium (Life Technologies, Inc., Grand Island, NY), 0.5 mg/ml bovine serum albumin, 20 mmol/L HEPES pH 7.4, 1 mmol/L phenylmethylsulfonyl fluoride, and 4 μg/ml leupeptin). The blocking buffer was then removed, and the sections were covered by a droplet of the same buffer containing 10 pmol/L ^{125}I-labeled rh-VEGF or ^{125}I-labeled rh-VEGF-C. Adjacent sections were incubated in the same concentration of iodinated growth factor in the presence of 1 nmol/L of the corresponding nonradioactive growth factor, to define nonspecific binding. Cross-competition of binding was assessed in the presence of 1 nmol/L rh-VEGF-C for ^{125}I-labeled VEGF or 1 nmol/L rh-VEGF for ^{125}I-labeled rh-VEGF-C binding.

After a 90-minute incubation in a humidified chamber at room temperature, the sections were rinsed five times (3 minutes each time) on ice, once with binding buffer and four times with phosphate-buffered saline. Sections were then fixed for 10 minutes in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 mol/L phosphate buffer pH 7.4, rinsed for 2 to 5 seconds in dH_20, and dried at room temperature for approximately 2 hours. The dried sections were covered with NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) and stored at 4°C for 2 weeks, developed, and stained.

Immunohistochemistry

Human skin from the leg, neck, and lower lip obtained after surgical removal or from buccal mucosa biopsies was frozen immediately, sectioned, stored at −70°C, and used for immunohistochemistry; one case of lymphangiomatosis and two cases of intramuscular hemangiomas were obtained in the same fashion. Paraffin-embedded biopsy specimens of six cases of lymphangiomatosis taken from the limb skin of young men were also studied.[21] The monoclonal antibodies (mAbs) used were against CD31 (platelet/endothelial cell adhesion molecule 1; DAKO Immunoglobulins, Glostrup, Denmark), an as-yet molecularly undefined endothelial antigen (PAL-E; Sanbio, Uden, The Netherlands), laminin (Sigma Chemical Co., St. Louis, MO), von Willebrand factor/factor VIII-related antigen (vWF, 6.3 μg/ml; DAKO Immunoglobulins), and mAb 9D9 developed against the extracellular domain of VEGFR-3 expressed in a baculovirus system.[22]

Adjacent 5-μm cryosections were air-dried and fixed in cold acetone for 10 minutes. The sections were incubated with blocking serum (5% normal horse serum) and then with anti-VEGFR-3 at a concentration of 1.1 μg/ml, anti-CD31 (diluted 1:200), anti-vWF (diluted 1:200), PAL-E (0.15 μg/ml), or anti-laminin (diluted 1:2000) for 2 h in a humid atmosphere at room temperature. A subsequent incubation for 30 minutes in biotinylated anti-mouse serum was followed by a 30-minute incubation using reagents of the Vectastain Elite avidin-biotin complex (ABC)/HPR kit (Vector Laboratories, Burlingame, CA). A 60-minute incubation with ABC was found to be optimal. Peroxidase activity was developed with 3-amino-9-ethyl carbazole (Sigma Chemical Co.) for 10 minutes. Finally, the sections were stained with hematoxylin. Negative controls were done by omitting the primary antibody, by using irrelevant primary antibody of the same isotype, or by blocking the anti-VEGFR-3 by overnight incubation with a 10-fold molar excess of the immunogen. Five-μm-thick sections of paraffin-embedded tissue from cutaneous lymphangiomatosis were deparaffinized and heated in a microwave oven in 10 mmol/L citrate buffer, pH 6.0, at 780 W for 5 minutes, followed by 450 W for 10 minutes. The sections were then incubated in methanol containing 30% H_2O_2 for 30 minutes and processed as the cryosections.

For differential staining of lymph and blood vessels, the following double-staining protocol was used. Frozen 4-μm sections were fixed in acetone; incubated with undiluted PAL-E mAb supernatant for 1 hour, biotinylated horse anti-mouse antibody (Vectastain, dilution 1:200) for 30 minutes,

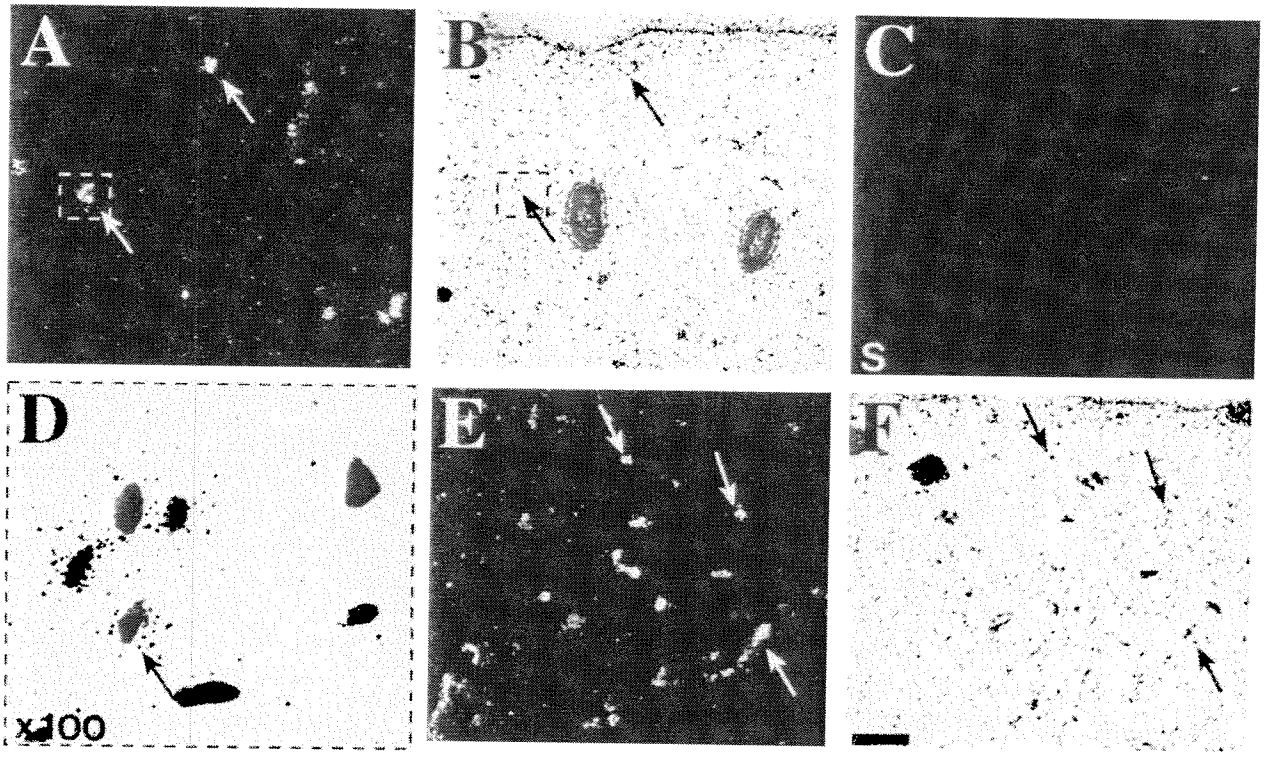

Figure 1. Expression of VEGFR-3 and VEGFR-2 in fetal skin analyzed by *in situ* hybridization. **A**, **B**, and **D**: Hybridization with the VEGFR-3 antisense probe. The signal originates from a subset of vessels of the dermis (**arrows**). **C**: Control hybridization with the VEGFR-3 sense strand. **D**: In a higher magnification of the area marked in (**A**) and (**B**), the autoradiographic grains are shown to be localized to the endothelial cells of a developing vessel. **E** and **F**: Hybridization for VEGFR-2. The probe gives signal in developing vessel structures at several levels of the dermis (**arrows**). **B**, **D**, and **F**: Bright-field photographs of the same sections. Bar: 0.1 mm.

and ABC-peroxidase (Vectastain, 1:100) for 45 minutes; and developed with 3-amino-9-ethyl carbazole for 10 minutes. For the second step, the sections were incubated with anti-CD31 mAb for 1 hour (1:2000), followed by incubation with rabbit anti-mouse immunoglobulin conjugated with alkaline phosphatase for 30 minutes (DAKO Immunoglobulins, 1:40), and developed with Fast Blue (Sigma Chemical Co.) for 20 minutes. All procedures were done at room temperature, and the sections were rinsed with phosphate-buffered saline between each step. In adjacent sections, VEGFR-3 expression was visualized by immunostaining with the 9D9 mAb, according to a procedure previously described for signal enhancement.[23] Briefly, acetone-fixed cryosections were incubated for 1 hour with anti-VEGFR-3 mAb 9D9, followed by biotinylated horse anti-mouse antibody for 30 minutes (1:200), ABC-peroxidase for 30 minutes (1:100), biotinylated tyramine solution (1:2000) containing 0.4 vol % of 30% H_2O_2 and ABC-peroxidase (1:100) for 20 minutes, and 3-amino-9-ethyl carbazole for 10 minutes, all at room temperature. Sections were counterstained with Harris' hematoxylin. After the staining procedures, all samples were examined by a trained pathologist.

Results

Localization of VEGF Receptors in Fetal Skin by in Situ Hybridization

To compare the expression of the VEGF-C receptors in normal fetal skin, analysis of their mRNAs was performed by *in situ* hybridization of adjacent sections. VEGFR-3 was expressed in putative developing lymphatic vessels mainly in the upper part of the developing dermis (Figure 1, A and B, arrows). Control hybridization with the probe from the VEGFR-3 sense strand did not give any specific signal above background (Figure 1C). When viewed at higher magnification, the cells showing the VEGFR-3 signal were often devoid of surrounding vessel wall structures; eg, no basement membrane could be identified around these cells (Figure 1D). VEGFR-2 showed a strong hybridization signal from numerous vessels located at various levels of the dermis (Figure 1, E and F).

Distribution of VEGF-C and VEGF Binding Sites in Adult Skin

To enable identification of the specific binding sites of VEGF-C and VEGF in the skin, iodinated recombinant human growth factors were used for *in vivo* ligand binding and autoradiography of frozen sections. The signal from the receptor-bound radioactive VEGF-C was visualized specifically in a horizontal zone of the subpapillary lymphatic plexus, which occurs in association with the superficial venous system at the junction of papillary and reticular dermis (Figure 2, A to C). These lymphatic vessels were seen as long extended structures, often collapsed, and had thin walls compared with the thicker walls of nearby blood vessels. Expression was also detected in the lymphatic vessels of the deep dermal plexus at the dermal-subcutaneous junction (data not shown). In

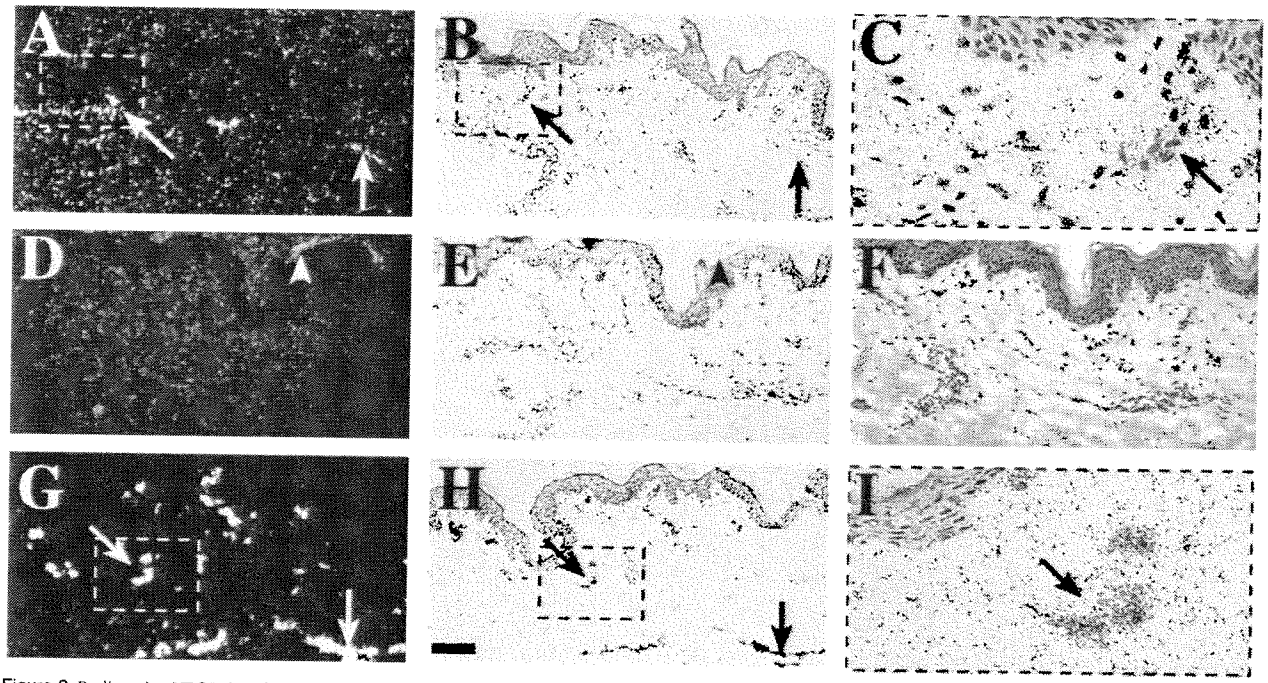

Figure 2. Radioactive VEGF-C and VEGF binding in human adult skin visualized by autoradiography. **A**, **B**, and **C**: Binding of ^{125}I-labeled rh-VEGF-C. The signal is localized to the thin lymphatic vessels of the subpapillary plexus (**arrows**). **D**, **E**, and **F**: Binding sites are blocked when a 100-fold excess of cold ligand is added to the incubation medium. Some unspecific binding is seen originating from keratin squames in the epidermis (**D**, **arrowhead**). **G**, **H**, and **I**: Specific ^{125}I-labeled rh-VEGF binding to vascular endothelium, which extends throughout the dermis. (**C** and **I**: Higher magnifications (×100) of the marked vessel structures binding the radioactive ligands VEGF-C and VEGF, respectively. Dark-field (**A**, **D**, and **G**) and bright-field (**B**, **C**, **E**, **F**, **H**, and **I**) exposures are shown. Bar: 0.1 mm for **A**, **B**, and **D** to **H**.

these sections, the binding was displaced with the addition of 1 nmol/L rh-VEGF-C to the incubation medium (Figure 2, D to F).

Incubation of the skin sections with iodinated VEGF revealed binding to all discernible vessels of every type throughout the dermis (Figure 2, G to I). In these sections, the binding was displaced from vessel endothelia with the addition of 1 nmol/L rh-VEGF. However, even in the presence of a 100-fold excess of cold VEGF, a weak signal was still detected in occasional cells that were not parts of the endothelial structures of the vessels. Unexpectedly, cross-competition of radioactive VEGF-C binding with VEGF or *vice versa* did not significantly change the binding patterns obtained (data not shown).

VEGFR-3 Immunohistochemistry

Staining of the fetal skin using the anti-VEGFR-3 mAb showed circular lymphatic structures in the corium layer of the developing dermis (Figure 3A, arrows). This pattern was similar to that seen in the *in situ* hybridization (compare with Figure 1, A and B). Adjacent skin sections were stained with anti-CD31 (Figure 3C), which decorated all endothelial cells, and PAL-E (Figure 3E), which detects endothelial cells in blood vessels. In adult skin, a few horizontally organized lymphatic vessels were stained by the anti-VEGFR-3 (Figure 3B, arrows). These vessels were difficult to trace in the adjacent sections stained by anti-CD31 (Figure 3D) and PAL-E (Figure 3F). According to previously published data, the PAL-E$^-$/CD31$^+$ vascular structures were defined as lymphatic vessels.[24] Indeed, comparison of the stainings confirmed that different subpopulations of the CD31-positive vessels were highlighted by anti-VEGFR-3 and PAL-E mAbs. To further confirm the identity of the lymphatic vessels, anti-VEGFR-3 staining of adult buccal mucosa was combined with double staining for CD31 and PAL-E.[24] This analysis directly confirmed that practically all vessels positive for VEGFR-3 were of the PAL-E$^-$/CD31$^+$ type (Figure 4, A and B). In addition to the blood vessel endothelium, PAL-E antibody also stained the basal lamina of the epidermis and buccal mucosa (Figures 3F and 4B, open triangles). In contrast to the blood vessels, the VEGFR-3-positive vessels had only very weak or no staining for laminin, which was used as a basal lamina marker (Figure 4D).[25]

VEGFR-3-Positive Vessels in Lymphangiomatosis and in Hemangioma

Lymphatic endothelium was also analyzed in six cases of the rare condition of lymphangiomatosis of the skin. Staining for VEGFR-3 was detected in thin endothelium-lined anastomosing channels dissecting through dermal connective tissue (Figure 5A). A negative control staining was done by blocking anti-VEGFR-3 with the immunogen (Figure 5B). The same structures in the adjacent section were vWF negative (Figure 5D, arrows), although blood vessels in the same section were vWF positive (Figure 5D, arrowheads). Both blood vessels (Figure 5C, arrowheads) and lymphatic vessels (Figure 5C, arrows) were

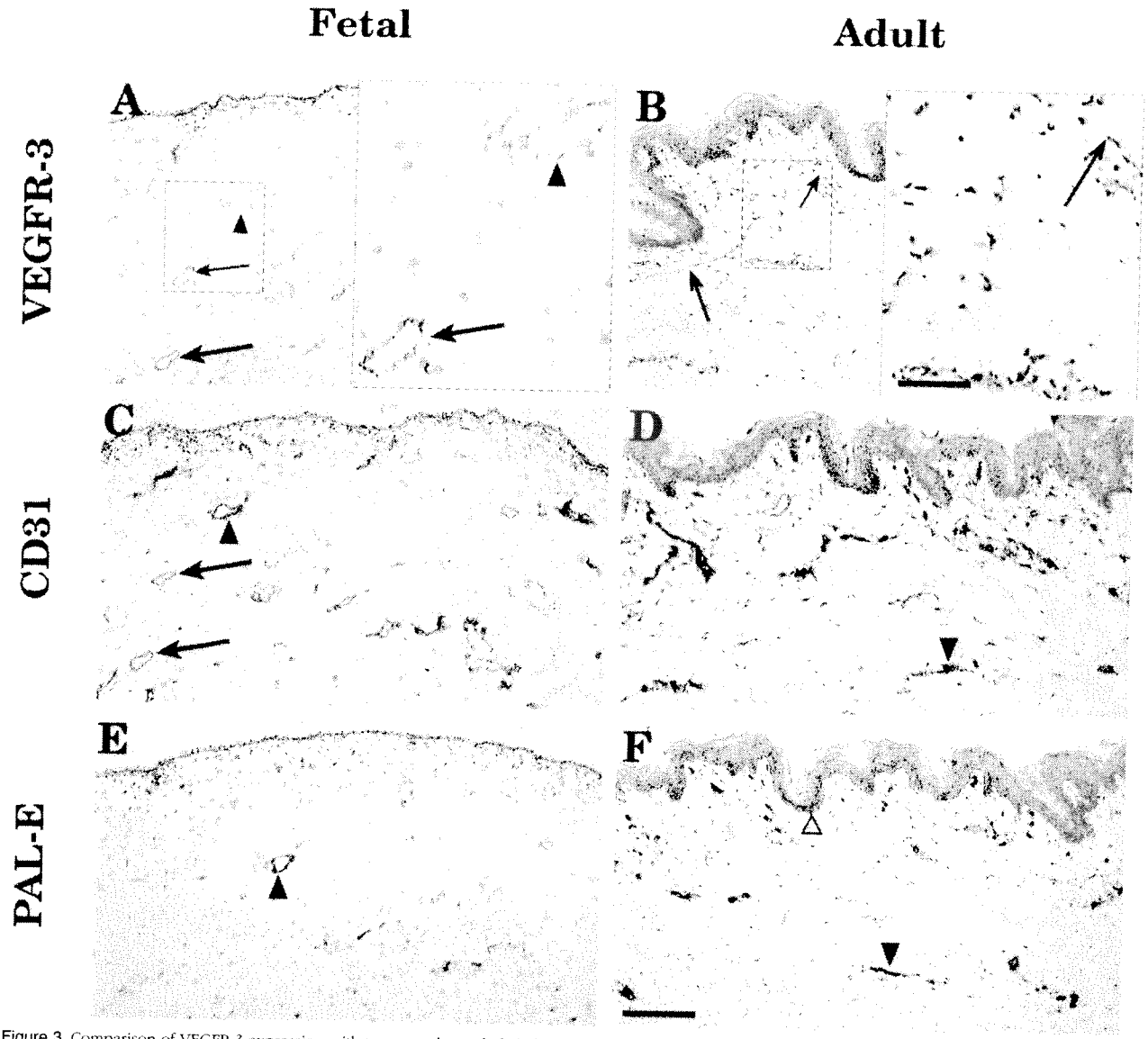

Figure 3. Comparison of VEGFR-3 expression with two vascular endothelial markers in fetal and adult skin. **Arrows** indicate vessels in adjacent sections of fetal and adult skin, respectively, which stain for VEGFR-3 (**A** and **B**) and CD31 (**C** and **D**), but not for PAL-E (**E** and **F**), and are therefore presumably lymphatic. **Arrowheads** identify vessels, which only react with antibodies against the two blood vascular endothelial antigens. In adult skin, the thin VEGFR-3-positive lymphatic vessels could be identified by morphology and lack of a basal lamina (**B**), but the same vessel could not be traced in the adjacent sections stained for CD31 (**D**) or PAL-E (**F**). Bar: 0.17 mm. **Insets** (**A** and **B**, **right**) illustrate higher magnifications of the lymphatic vessels (**arrows**) in marked areas of the fetal and adult skin sections, respectively. Bar: 57 μm.

stained for CD31. In three fixed, paraffin-embedded cutaneous capillary hemangiomas, endothelial cells lining the blood capillaries containing red cells and also endothelial cells apparently not associated with capillaries had very little or no VEGFR-3 signal, but were clearly positive for CD31 (Figure 5, E and F, arrowheads). Only some vessels, which were devoid of red cells and thus presumably lymphatic, expressed VEGFR-3 in the tissue surrounding the capillary hemangiomas (Figure 5E, arrows). However, when adjacent cryosections of two intramuscular hemangiomas were stained for VEGFR-3 (Figure 5G) and for the endothelial marker CD31 (Figure 5H), the majority of the CD31-positive vessels with lumens were found to express at least some VEGFR-3-positive staining (Figure 5H, arrows). The identity of such vessels is unknown at present.

Discussion

A uniformly structured, dense subpapillary, and wide-meshed deep dermal lymphatic network is a common feature of all skin regions. Although a reliable detection technique has been developed to demonstrate dermal lymph vessel networks in skin samples,[26] a specific marker for lymphatic endothelial cells would be useful, eg, in the diagnosis of lymphangiomas. Here we present such a marker, the VEGFR-3, using mAbs and show that

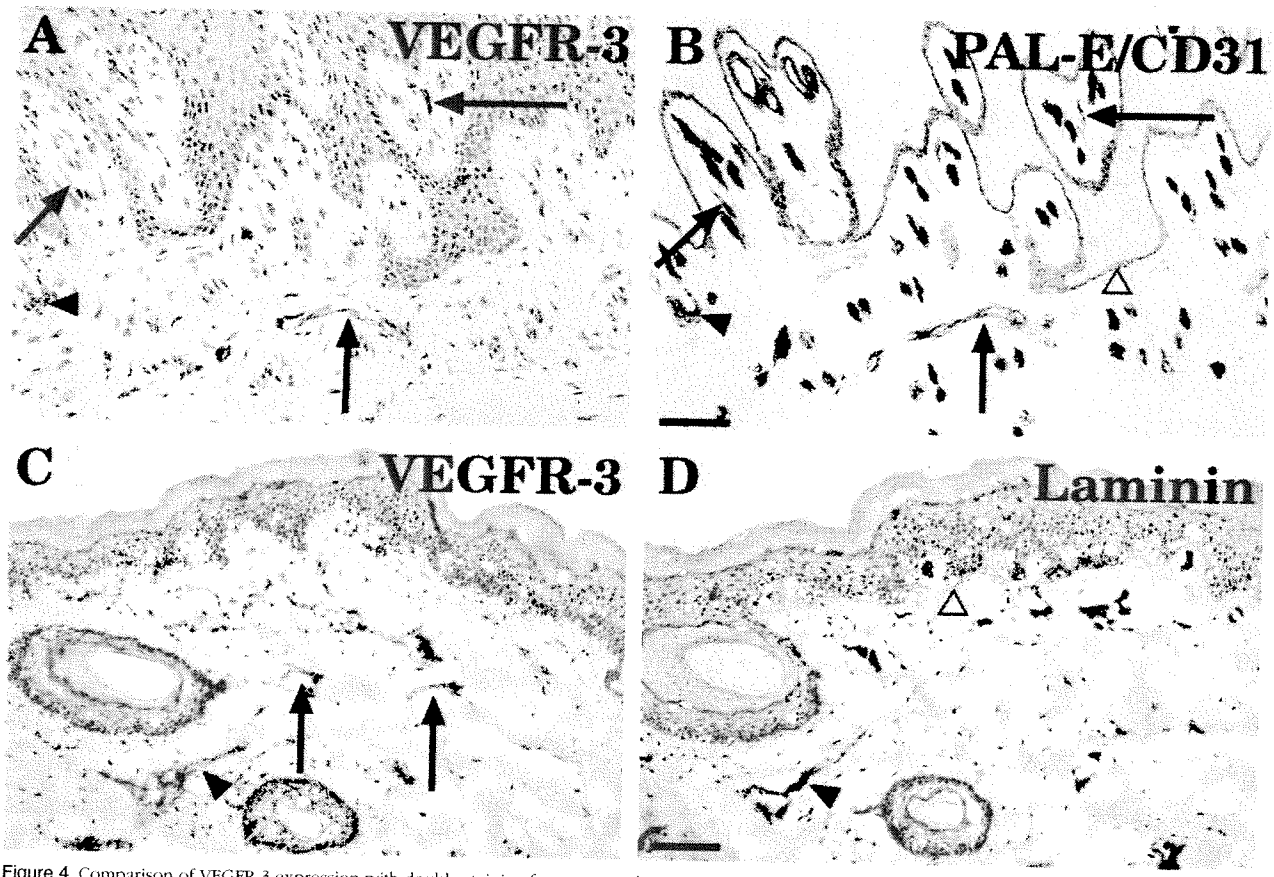

Figure 4. Comparison of VEGFR-3 expression with double staining for two vascular endothelial markers and with anti-laminin staining. A sample of adult buccal mucosa was stained with the anti-VEGFR-3 mAb (**A**) and an adjacent section, first with PAL-E (**B**, red-colored endothelia) and then with anti-CD31 (**B**, blue-colored endothelia), to distinguish lymphatic structures from blood vessels. PAL-E also decorates the epidermal basal lamina (**B**, open triangle). Comparison of VEGFR-3 and laminin expression in adult skin is shown in (**C**) and (**D**). **Arrows** indicate lymphatic vessels of adult skin, which stain for VEGFR-3 (**C**). **Arrowheads** show blood vessels with basement membranes, which stain for laminin (**D**), but only very weakly for VEGFR-3. Bars: 35 μm (**A** and **B**) and 0.11 mm (**C** and **D**).

it identifies the VEGF-C growth factor receptor and a distinct vessel population both in fetal and adult skin, which has properties of lymphatic vessels.

The mRNA expression patterns of the VEGFRs were distinct but partially overlapping in the fetal skin analyzed by *in situ* hybridization. Expression of VEGFR-3 appeared to be restricted to a subpopulation of vessels in the upper dermis, which may correspond to the developing superficial lymphatic plexus of the fetal skin. The mRNA signal for the major mitogenic VEGF receptor, VEGFR-2, was observed in all vessels throughout the developing skin. This finding is in agreement with a previous report that the distribution of these receptors in different fetal organs partially overlaps, yet certain endothelia lack one or two of the three receptors.[19] These results are also consistent with the suggested specific roles of each of these receptors in the vascular system of the skin.

The results of iodinated ligand binding experiments are consistent with the results obtained by *in situ* hybridization. The iodinated VEGF-C was shown to bind preferentially to the lymphatic vessels in which VEGFR-3 was shown to be expressed by *in situ* hybridization. These results are in agreement with earlier findings that VEGFR-3 expression was shown to become restricted to the lymphatic vessels in adult mouse tissues and some high endothelial venules in adult human tissues.[27] However, skin was not examined in the previous study. Somewhat surprisingly, although VEGF-C and VEGF compete for VEGFR-2 binding, ^{125}I-labeled rh-VEGF-C binding to the skin was not detectably decreased by the addition of cold VEGF (unpublished data), suggesting that in this tissue sample, VEGF-C bound mostly to VEGFR-3. This cannot be fully explained with the threefold-greater affinity of VEGF-C toward VEGFR-3 when compared with binding to VEGFR-2.[20] However, a 100-fold excess of VEGF-C abolished completely VEGF-C-specific binding to its receptor(s).

In the case of ^{125}I-labeled rh-VEGF, binding was detected in most of the vessel structures, including those that bound radioactive VEGF-C. VEGF has been reported to have about a 5- to 10-fold greater affinity for VEGFR-1 than to VEGFR-2, so that the observed signal in this experiment could originate mostly from binding to VEGFR-1.[13,28–30] Thus, if VEGFR-1 were expressed in the same endothelia, blocking of VEGFR-2 sites with VEGF-C would not necessarily change the binding patterns observed. On the other hand, as VEGF is shown to compete more efficiently for VEGFR-2 binding than VEGF-C, added VEGF-C might not be able to compete for all receptor sites, and this would leave some signal unquenched.[20]

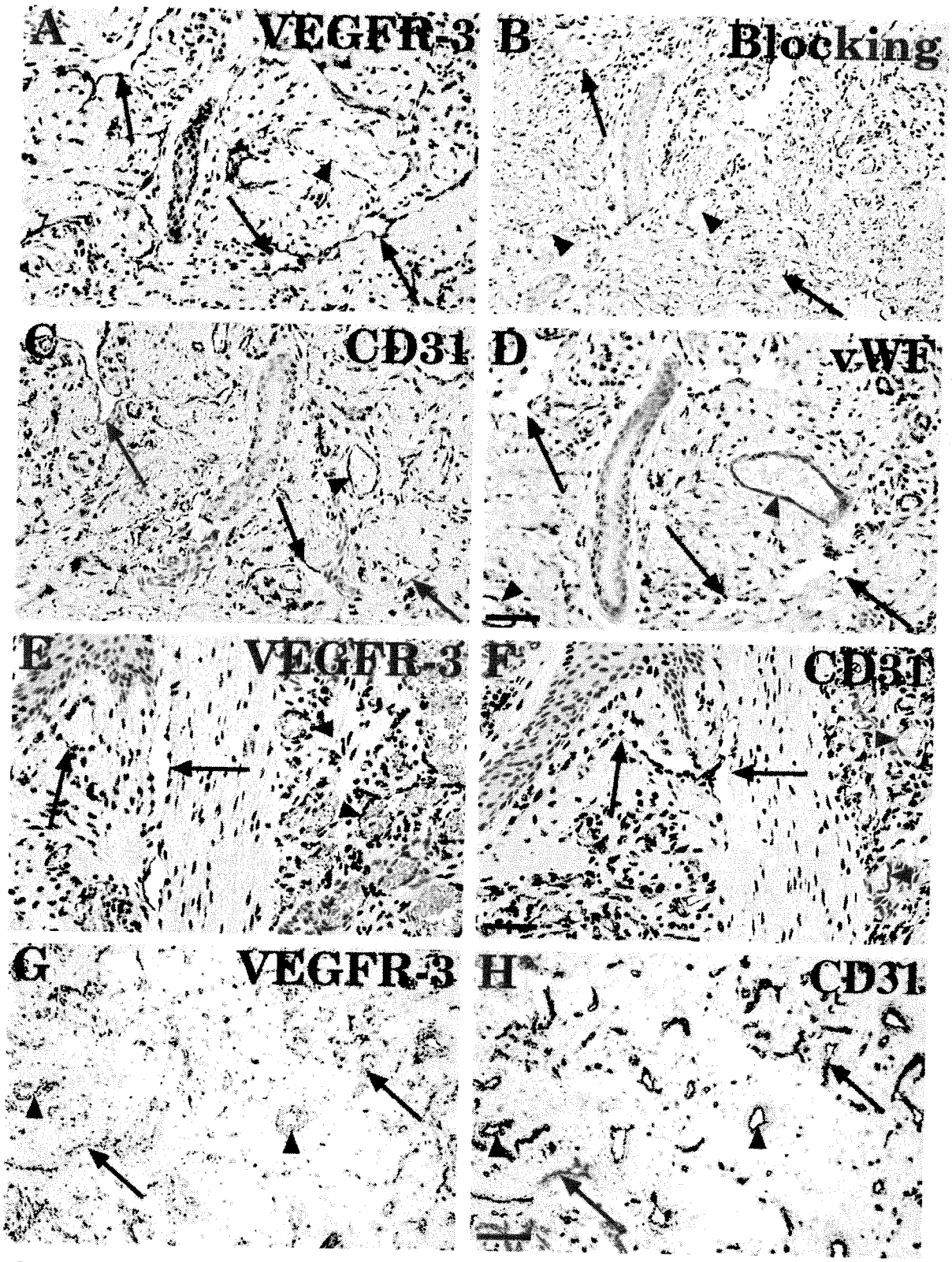

Figure 5. Comparison of VEGFR-3 expression with vascular endothelial markers in lymphangiomatosis (A to D) and in capillary cutaneous (E and F) and intramuscular (G and H) hemangiomas. Arrows indicate collapsed, VEGFR-3-positive thin endothelium-lined anastomosing channels dissecting through dermal connective tissue in a lymphangiomatosis sample (A). Negative control was done by blocking anti-VEGFR-3 with the immunogen (B). Adjacent sections were also stained for CD31 (C) and vWF (D). Note that in contrast to the buccal mucosa sample (Figure 4), CD31 identifies the endothelial cells lining lymphatic spaces in lymphangiomatosis, whereas the vWF staining is weak or absent. In cutaneous hemangioma, the well-formed capillaries with prominent endothelial cells show very little or no expression of VEGFR-3 (E), but are strongly CD31 positive (F). In a frozen section of intramuscular hemangioma, weak VEGFR-3 staining is detected (G, arrows) in part of the CD31-positive vessels (H). Bars: 65 μm (A to D, 48 μm (E and F), and 140 μm (G and H).

Immunohistochemical staining of fetal and adult skin samples showed that anti-VEGFR-3 mAbs identify a subpopulation of vessels positive for the pan-endothelial marker CD31, but negative for PAL-E, which has been previously suggested to define lymphatic vessels.[24] The lack of staining for the basal lamina antigen laminin was consistent with such a conclusion. Cutaneous lymphangiomatosis was chosen for immunohistochemistry, because the disorder is characterized by proliferation of presumed lymphatic endothelium.[21] Besides the skin, lymphangiomatosis often involves bone, soft tissue, or viscera during the first 20 years of life.[31] Whereas the dermal lymph vessels were relatively weakly stained in healthy adult skin using the anti-VEGFR-3, the endothelia of lymphangiomatosis lesions were strongly stained. This is consistent with earlier results showing enhanced VEGFR-3 mRNA expression in lymphangiomas.[27] Furthermore, these results support the theory that the lymphangiomatosis lesions indeed develop from aberrant rests of lymphatic tissue with obliteration of draining lymphatics.[32]

Capillary hemangioma is a benign acquired vascular tumor, consisting of multiple thick- and thin-walled vascular structures.[33] It occurs frequently in the skin and sometimes in the internal organs. Compared with normal capillaries, the vessels in capillary hemangiomas have a more prominent endothelial lining. The antibody against VEGFR-3 gave little or no staining of CD31-positive endothelial cells decorating blood vessels in fixed, paraffin-embedded samples of cutaneous capillary hemangiomas. Also, the scattered endothelial cells or clusters of them in well-formed collagen stroma contained very little or no VEGFR-3. However, weak staining of small vessels was obtained in two frozen sections of intramuscular hemangioma lesions, suggesting that these lesions contain endothelial cells in vessels that share phenotypic properties with the lymphatic vessels. One possibility is that such vessels are less differentiated than mature blood vessels, thus resembling early embryonic vessels, which express all three VEGFRs.[27–30]

In summary, it may be concluded on the basis of specific radioactive ligand binding, receptor *in situ* hybridization, and immunohistochemistry that VEGFR-3 is distributed in a manner consistent with the known lymphatic vascular pattern in human skin and that the anti-VEGFR-3 mAbs should prove useful in studies of skin diseases affecting the lymphatic or blood vascular system.

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